REMARKS

Claims 1-2 have been amended to limit the claims to the infection of mammalian cells or tissue. Claims 7-8 have been canceled without prejudice in accordance with the restriction requirement. Thus, claims 1-6 are currently pending. The amendments are fully supported by the specification and original claims and do not contain new matter.

The Applicants expressly rebut any presumption that the Applicants have surrendered any equivalents under the doctrine of equivalents and expressly state that the claims, as amended, are intended to include and encompass the full scope of any equivalents as if the claims had been originally filed and not amended.

I. Claim Rejections Under 35 USC § 112

The claims have been amended deleting the term "in vitro" from the claims which was inserted into the claims via the previous amendment (without prejudice) filed June 20, 2005 rendering a previous enablement rejection moot. The Applicants have now amended the claims as they were originally filed without the "in vitro" limitation and present the following reasons of why such claims are enabled and should not be rejected under 35 USC § 112.

Under MPEP § 2107.03(III), data from *in vitro* testing is sufficient to support therapeutic utility under either the utility or the enablement requirement. *See* MPEP § 2107.01 "Relationship Between 35 U.S.C. 112, First Paragraph, And 35 U.S.C. 101" which explains that the utility portion of the enablement requirement under 35 U.S.C. 112 requirement is the same as the utility requirement under 35 U.S.C. 101 ("a rejection based on 'lack of utility,' whether grounded upon 35 U.S.C. 101 or 35 U.S.C. 112, first paragraph, rests on the same basis (i.e., the asserted utility is not credible)").

With respect to establishing the enablement or utility of a method of inhibiting gene expression in culture (*in vitro*) v. in an organism (*in vivo*), MPEP § 2107.03 states:

> **III. DATA FROM *IN VITRO* . . . TESTING IS GENERALLY SUFFICIENT TO SUPPORT THERAPEUTIC UTILITY**
> [D]ata generated using *in vitro* assays, or from testing in an animal model or a combination thereof almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process. A cursory review of cases involving therapeutic inventions where 35 U.S.C. 101 [or 35 U.S.C. 112] was the dispositive issue illustrates the fact that the Federal courts are not particularly receptive to rejections under 35 U.S.C. 101 [or 35 U.S.C. 112] . . . . Evidence does not have to be in the form of data from an art-recognized animal model for the particular disease or disease condition to which the asserted utility relates . . . . Office personnel should be careful not to find evidence unpersuasive simply because no animal model for the human disease condition had been established prior to the filing of the application. See *In re Chilowsky*, 229 F.2d 457, 461, 108 USPQ 321, 325 (CCPA 1956) ("The mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it."); *In re Wooddy*, 331 F.2d 636, 639, 141 USPQ 518, 520 (CCPA 1964) ("It appears that no one on earth is certain as of the present whether the process claimed will operate in the manner claimed. Yet absolute certainty is not required by the law. The mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it.") (emphasis added).

Here, the data in the current application was generated by infecting cells or tissue in culture with two separate sets of viral particles. It is well known in the art that nucleic acids may be delivered to cells *in vivo* by viral infection (e.g., gene therapy). Thus, a utility established by infecting cells in culture *in vitro* clearly supports a reasonable correlation for its use *in vivo* and thus satisfies the enablement requirement. Again, there is no requirement that the Applicants provide *in vivo* data for the enablement of an *in vivo* method ("data generated using *in vitro* assays almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process" MPEP § 2107.03(III)).

Thus, for the above reasons, it is clear that the claims (as amended) should not be rejected under 35 USC § 112 or 101 for lack of enablement or utility based on the lack of *in vivo* examples.

II. Claim Rejections Under 35 USC § 103

The Examiner has rejected claims 1-6 under 35 U.S.C. § 103(a) as being unpatentable over Heifetz (WO 99/61631) in view of Lundstrom. According to the Examiner, Heifetz teaches a process of inhibiting the expression of a gene in a plant cell by administering a sense RNA fragment and an antisense RNA fragment, which are administered into the cell sequentially (citing p. 7). The Examiner also states that Heifetz teaches the use of "viral vectors" to introduce RNA fragments into plant cells (citing page 11). The Examiner further cites Lundstrom for teaching "alphavirus vectors."

A. No Prima Facie Case of Obviousness Has Been Established

First, a *prima facie* case of obviousness has not been established because: (a) Heifetz (taken alone or in combination with Lundstrom) does not teach or suggest all the claim limitations; and (b) there is absolutely no motivation to modify Heifetz to obtain the presently claimed invention.

1. Heifetz Alone Or In Combination With Lundstrom Does Not Teach Or Suggest All Of The Claim Limitations

Heifetz (taken alone or in combination with Lundstrom) does not teach or suggest all the claim limitations of each claim. Under MPEP § 2143.03 in order to establish a *prima facie* case of obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art. If the prior art references do not disclose or suggest all of the claim limitations either by themselves or in combination with each other, there can be no *prima facie* case of obviousness.

Here, neither Heifetz or Lundstrom teach or suggest at least two important limitations common to all of the claims of the present invention which are: (1) infecting cells or tissue with a set of viral particles expressing sense RNA and (2) infecting cells or tissue with a separate set of viral particles expressing anti-sense RNA.

Although the Examiner concludes that a virus is used in Heifetz to introduce RNA molecules into plant cells, e.g. by "agroinfection" (citing page 11 of Heifetz), this conclusion is incorrect. Heifetz only teaches the use of "viral vectors" to introduce RNA fragments into plant cells. Heifetz does not disclose the use of viruses to introduce such RNA. The Applicants respectfully note that "viruses" and "viral vectors" are completely different. Although Heifetz on p. 14 ambiguously states that "examples of self-replicating vectors are viruses," this statement is completely incorrect and completely contrary to the meaning of the term. A vector is clearly defined as "the DNA of an agent (virus or plasmid) used to transmit genetic material to a cell or organism." Molecular Biology of The Cell, 4th edition (2002), p. G:36 of the Glossary. Vectors are physically constructed of nucleic acids. A vector is not the viral particle itself (a virion or virus) that is constructed of a protein coat that encapsulates the nucleic acid. Heifetz does not disclose the use of viruses or viral particles as a method of introducing two separate sets of RNA fragments into plant cells (one sense and the other antisense).

More specifically, on page 11 of Heifetz it clearly states that "viral vectors are used to introduce DNA molecules or RNA fragments of the present invention into plant cells, e.g. through so-called agroinfection." However, the term "agroinfection" means that the **bacterium** *Agrobacterium tumefaciens* is used to introduce a viral vector into a plant cell- not a virus. *See* Leiser et al, *Agroinfection as an alternative to insects for infecting plants with beet western yellows luteovirus*, PROC. NATL. ACAD. SCI. Vol. 89, pp. 9136-9140 (October 1992), which states "Agroinfection is a procedure for introducing a plant virus into a host via *Agrobacterium tumefaciens* (see abstract). A copy of the Leiser et al. article is enclosed for the Examiner's convenience.

The only method of transferring the "viral vectors" containing the RNA fragments of Heifetz into host cells [(sense or antisense) and (sequentially or not sequentially)] is through transformation such as PEG-mediated transformation, agroinfection, particle bombardment, electroporation, or microinjection. *See* page 7, third full paragraph, of Heifetz which states that "the RNA fragments are introduced in the plants cell by different transformation methods. For example, the RNA fragments are transferred to the host cells using particle bombardment . . . . PEG-mediated transformation . . . electroporation . . . . [and] microinjection of the RNA fragments . . ." *See also* page 11 of Heifetz which states that the RNA fragments may be introduced into the plant cell through "agroinfection" (which as stated above means that the **bacterium** *Agrobacterium tumefaciens* is used to introduce a viral vector into a plant cell- not a virus). In addition, not one of the nine examples disclosed in Heifetz disclose the use of two separate sets of viral particles (one containing sense and the other antisense RNA) to infect host cells or tissue. Although example 5 mentions that a RNA or DNA virus containing a inducible recombinase expression cassette may be used to infect plant cells containing the desirable transgene construct, this virus is only used to introduce the gene encoding the enzyme "recombinase" into the cell (but not RNA sense and anti-sense strands which will inhibit expression of a target gene in the plant cell). Heifetz does not disclose the introduction of RNA sense and anti-sense strands into plant cells using a virus.

Accordingly, it is clear from the disclosure of Heifetz that Heifetz does not teach or suggest the introduction of RNA sense and anti-sense strands into plant cells using a virus. In contrast, Heifetz teaches away from using viruses as the delivery method by instead focusing heavily on transformation methods using the bacterium *Agrobacteriuim tumefaciens*, particle bombardment, protoplast uptake (e.g., PEG-mediated and electroporation), microinjection, and pollen transformation. *See* the last paragraph on p. 39 of Heifetz.

In sum, the use of viruses or viral particles to infect host cells to deliver RNA sense and anti-sense strands is not disclosed or suggested anywhere in Heifetz (i.e., "viral vectors" are completely different from viral particles); and Heifetz especially does not teach or suggest a method of (1) infecting cells or tissue with one set of viral particles expressing sense RNA and (2) infecting cells or tissue with a second or separate set of viral particles expressing anti-sense RNA. Combining Lundstrom with Heifetz does nothing to cure this fatal defect in the disclosure of Heifetz. Thus, claims 1-6 are not obvious for this reason alone.

2. There is No Motivation To Modify Heifetz Or To Combine Lundstrom With Heifetz

There is absolutely no motivation to modify Heifetz (WO 99/61631) to obtain the presently claimed invention or to combine Lundstrom with Heifetz. "To establish a *prima facie* case of obviousness . . . there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference." *See* MPEP § 2143.01, entitled "Basic Requirements of a *Prima Facie* Case of Obviousness." Under MPEP §2143.01, "[t]he mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination" and the "fact that the claimed invention is within the capabilities of one of ordinary skill in the art is not sufficient by itself to establish prima facie obviousness . . . . The level of skill in the art cannot be relied upon to provide the suggestion to combine [or modify] references").

Heifetz (WO 99/61631) describes a method to alter the expression of a target gene in a plant whereas the present invention (as amended) is directed to a method of inhibiting mammalian cells or tissue. Plant cells have in addition to the cell membrane a cell wall mainly consisting of cellulose whereas mammalian cells have only a cell membrane. Due to this fundamental structural difference between plant cells and mammalian cells with regard to the cell envelope (cell wall for plant cells and cell

membrane for mammalian cells), a person skilled in the art would not consider a method for the introduction of RNA into plant cells as a starting point to develop a method for inhibiting a target gene by the introduction of RNA into mammalian cells or tissue. Therefore, no one skilled in the art would be motivated to modify Heifetz or to combine Heifetz with Lundstrom to arrive at the presently claimed invention. Accordingly, claims 1-6 are not obvious for this additional reason.

Moreover, as stated above, combining Heifetz with Lundstrom does not cure the deficiencies of Heifetz (all the claim limitations would still not be taught or suggested by the combination). Thus, even if a motivation was found to combine Heifetz with Lundstrom, Lundstrom adds nothing with respect to a motivation to cure the deficiencies of Heifetz (as stated above).

Accordingly, no motivation has been established to modify Heifetz to obtain the present invention. Thus, a *prima facie* case of obviousness has not been established for all of the above reasons.

B. There Are Surprising And Unexpected Results

The Examples of the present application surprisingly demonstrate that when the sense and anti-sense fragments are provided by different viral particles there is an inhibition of the expression of chromosomal cyclin genes. *See* Example 6 and Figure 6 of the present invention). In contrast, when both sense and anti-sense fragments are provided in the same construct in the same viral particle inhibition of the expression of chromosomal cyclin genes does not occur (*see* Example 7 of the present invention). Under MPEP § 2144-45, evidence of such surprising and unexpected results rebuts a *prima facie* case of obviousness.

More specifically, all of the claims of the present invention require that cells or tissue be infected by two different sets of viral particles: one set providing the sense

RNA and the other providing the anti-sense RNA. As shown in Example 7 (page 15 of the specification), when both sense and anti-sense sequences are cloned into the same construct in the same viral particle, there was no inhibition of the target gene(s) after infection. But as shown in Example 6, when the sense and anti-sense sequences are cloned separately into separate viral particle populations, there was inhibition of the target gene(s) after infection, *see* page 15 of the specification). This represents surprising and unexpected results.

In contrast, Heifetz (WO 99/61631) does not necessarily require that the sense and antisense fragments be administered separately. *See* Heifetz on page 7, last paragraph, which states, "the RNA fragments are mixed before being introduced into said cell [or alternatively] . . . . the RNA fragments are introduced sequentially." If the first method of Heifetz is employed (mixing the sense and antisense before introduction into the cell), then no inhibition of gene expression would occur based on the results of Applicants' Example 7. Thus, in essence, because Heifetz does not make this important distinction, Heifetz is nonenabling and inoperative prior art, which renders it unsatisfactory for its intended purpose. *See* MPEP § 2143.01(V-VI) and § 2145(X). Heifetz does not distinguish or even remotely recognize or suggest the surprising and unexpected results provided by Applicants' Examples which show that the RNA fragments must be introduced into the cell by viruses separately in order to obtain inhibition of expression.

Accordingly, even if a *prima facie* case of obviousness had been established (which it hasn't), these surprising and unexpected results would rebut any assertion that the claimed invention is obvious.

For all of the above reasons, the Applicants respectfully request that the rejections under 35 U.S.C. § 103 be withdrawn.

Conclusion

Entry of the foregoing remarks and amendments is respectfully requested.

If the Examiner disagrees with the above, then the Applicants respectfully request that the rejections be made final in the next Office Action at which time the Applicants will file a Notice of Appeal and submit an Appeal Brief.

If any additional fee is deemed necessary, authorization is given to charge the amount of any such fee to Deposit Account No. 08-2525.

Respectfully submitted,

Attorney for Applicant(s)
Brian C. Remy
(Reg. No. 48176)
340 Kingsland Street
Nutley, NJ 07110
Telephone (973) 235-6516
Telefax: (973) 235-2363

259051

Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 9136–9140, October 1992
Plant Biology

Agroinfection as an alternative to insects for infecting plants with beet western yellows luteovirus

R.-M. LEISER*†, V. ZIEGLER-GRAFF‡§, A. REUTENAUER‡, E. HERRBACH¶, O. LEMAIRE¶, H. GUILLEY‡, K. RICHARDS‡, AND G. JONARD‡

*Institute for Genetics and Crop Research, 0-4325 Gatersleben, Federal Republic of Germany; ‡Institut de Biologie Moléculaire des Plantes du Centre National de la Recherche Scientifique et de l'Université Louis Pasteur, 12 rue du Général Zimmer, 67084 Strasbourg, France; and ¶Station de Recherches 'Grandes Cultures', Institut National de la Recherche Agronomique, 68021 Colmar, France

Communicated by Robert J. Shepherd, June 18, 1992 (received for review April 3, 1992)

ABSTRACT **Beet western yellows luteovirus, like other luteoviruses, cannot be transmitted to host plants by mechanical inoculation but requires an aphid vector, a feature that has heretofore presented a serious obstacle to the study of such viruses. In this paper we describe use of agroinfection to infect hosts with beet western yellows virus without recourse to aphids. Agroinfection is a procedure for introducing a plant virus into a host via *Agrobacterium tumefaciens* harboring a Ti plasmid, which can efficiently transfer a portion of the plasmid (T-DNA) to plant cells near a wound. The viral genome must be inserted into the T-DNA in such a way that it can escape and begin autonomous replication, a requirement that has, so far, limited agroinfection to pathogens with a circular genome. We have cloned cDNA corresponding to the complete beet western yellows virus RNA genome between the cauliflower mosaic virus 35S promoter and the nopaline synthase transcription termination signal. In one construct, a self-cleaving (ribozyme) sequence was included so as to produce a transcript *in planta* with a 3′ extremity almost identical to natural viral RNA. When inoculated mechanically to host plants, the naked plasmid DNA was not infectious but, when introduced into T-DNA and agroinfected to plants, both the construct with and without the ribozyme produced an infection. This approach should be applicable to virtually any plant virus with a linear plus-strand RNA genome.**

The luteoviruses are a large group of plant viruses with a monopartite plus-strand RNA genome packaged in small isometric virions (for reviews, see refs. 1 and 2). They are transmitted in a persistent circulative fashion by several aphid species and are generally confined to host phloem tissues. Luteovirus RNA and virions cannot be transmitted by mechanical inoculation. Recently, the genomes of several luteoviruses have been characterized (3–6), and *in vitro* transcripts of full-length cDNA clones of barley yellow dwarf virus and beet western yellows virus (BWYV) have been shown to be infectious in protoplasts (7, 8). These transcripts lend themselves to the study of gene function by "reverse genetics," in which specific mutations are introduced into the cloned cDNA and the biological properties of transcripts carrying the mutations are investigated. However, the aforesaid nontransmissibility of luteoviruses to whole plants by mechanical inoculation impedes many interesting applications of this approach.

Agrobacterium tumefaciens harboring a Ti plasmid can efficiently transfer a portion of this plasmid called the T-DNA to plant cells in the vicinity of a wound (9). This property has provided the basis for a technique called "agroinfection" or "agroinoculation" in which a viral genome is inserted into the T-DNA in such a way that it can escape and initiate an infection once within a target cell (10). Up until now, agroinfection has been limited to infectious agents with circular genomes [i.e., cauliflower mosaic virus (CaMV), geminiviruses, and potato spindle tuber viroid (for review see ref. 11)]. In this paper we describe an agroinfection system suitable for a plant virus with a linear single-stranded RNA genome and its use to infect plants with cloned BWYV cDNA.

MATERIALS AND METHODS

Plasmid Construction. The previously described full-length clone of BWYV isolate FL1 cDNA (pBW_0; ref. 8) served as the source for all viral sequences. *Escherichia coli* strain SURE (Stratagene) was used as host for constructions. A CaMV 35S promoter cassette (ref. 12; and further modifications), containing nucleotides (nt) −417 to −1 relative to the CaMV transcription initiation site, was introduced into pBluescript SK(−) (Stratagene) to produce pBK35. pBK35 contains a unique *Stu* I site with the point of cleavage coincident with the transcription initiation site (12). A DNA fragment (P1; Fig. 1*A*) containing nt 1–376 of the BWYV sequence was synthesized by PCR. The upstream primer, 5′-CCG*GTCGACAAAAGAAACCAGGAGGGA*, contained a *Hin*cII site (italicized) overlapping the 5′ terminal viral sequence (underlined). P1 was cut with *Hin*cII and *Xho* I (nt 256), and the resulting fragment was inserted between the *Stu* I and *Xho* I sites of pBK35 to give p35BW51 (Fig. 1*A*). A *Bam*HI–*Xho* I fragment from p35BW51 containing the promoter and cDNA insert was then cloned between the *Bam*HI–*Xho* I sites of pRT103 (14) to give p35BW52. The insert region was excised from p35BW52 as a *Bam*HI–*Hin*dIII fragment and placed between the *Bam*HI–*Hin*dIII sites of pUC18S (a derivative of pUC18 with an additional *Sal* I site preceding the polylinker *Hin*dIII site) to give p35BW53. The foregoing steps were necessary to obtain an appropriate configuration of polylinker sites. The rest of the BWYV sequence (4) was inserted in two steps as an *Xho* I–*Eco*RV fragment (nt 256–1412) between the *Xho* I–*Hin*cII sites of p35BW53 and as a *Spe* I–*Sal* I fragment (nt 1350–5641) between the *Spe* I and polylinker *Sal* I sites to produce $pBW.A^+$ (Fig. 1*A*).

The 3′ poly(A) tail of $pBW.A^+$ was replaced by *Xba* I and *Sst* I sites by oligonucleotide-directed mutagenesis (8) to yield $pBW.A^-$ (Fig. 1*A*), and an *Sst* I–*Sal* I fragment containing the nopaline synthase (NOS) transcription termination signal and polyadenylylation site (15) was inserted between the vector *Sst* I and *Sal* I sites to create pBW.N (Fig. 1*A*). The ribozyme sequence was provided as a 54-mer

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: BWYV, beet western yellows virus; CaMV, cauliflower mosaic virus; NOS, nopaline synthase; nt, nucleotide(s); T-DNA, transferred DNA.

†Present address: Diagen GmbH, Max-Volmer-Strasse 4, W-4010 Hilden, F.R.G.
§To whom reprint requests should be addressed.

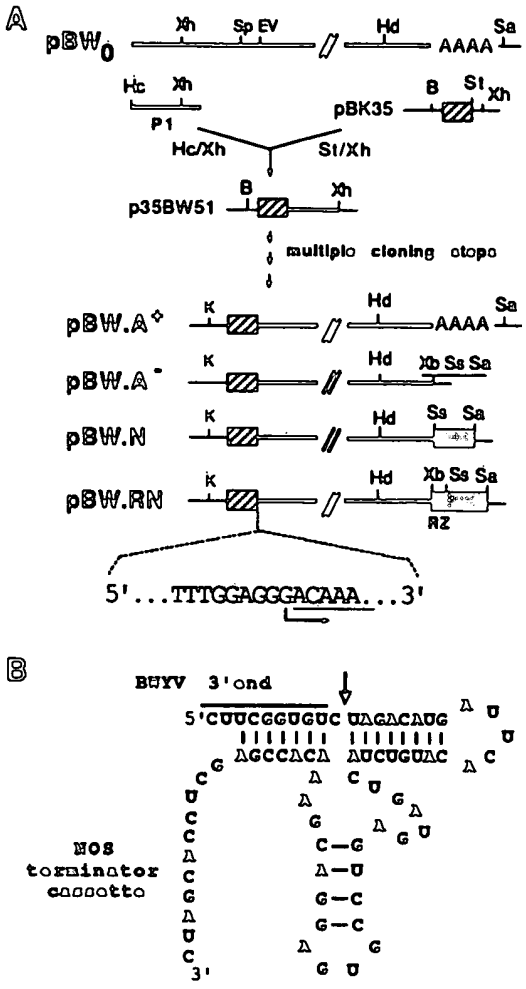

FIG. 1. Structure of clones used in agroinfection. (*A*) A simplified scheme for construction of pBW.N and pBW.RN. Thick lines represent BWYV cDNA sequences (not to scale), and thin lines represent flanking vector sequences. Hatched rectangle, 35S promoter cassette; black rectangle, ribozyme (RZ) cassette; cross-hatched rectangle, NOS terminator cassette. The sequence at the 35S promoter–viral cDNA junction shows the transcription initiation site (arrow) and the beginning of the viral sequence (underlined). Relevant restriction sites are *Bam*HI (B), *Eco*RV (EV), *Hin*cII (Hc), *Hin*dIII (Hd), *Kpn* I (K), *Sal* I (Sa), *Spe* I (Sp), *Stu* I (St), *Sst* I (Ss), *Xba* I (Xb), and *Xho* I (Xh). (*B*) Structure of the ribozyme separating the BWYV 3′ terminus from the NOS terminator cassette. The sequence of the nonviral portion of the ribozyme sequence was based on the consensus hammerhead structure given in ref. 13. The arrow indicates the predicted site of self-cleavage.

(5′-CTAGACATGATTCACATGTCTACTGATGAGTC-CGTGAGGACGAAACACCGAGCT) hybridized with a 46-mer complementary to all but the underlined extremities of the 54-mer. The hybrid was inserted between the *Xba* I and *Sst* I sites of pBW.A⁻ to produce pBW.R. The NOS cassette was then introduced into pBW.R to yield pBW.RN (Fig. 1*A*).

Transcription vectors p3′BW.N and p3′BW.RN were obtained by placing the *Hin*dIII–*Sal* I fragment from pBW.N and pBW.RN between the *Hin*dIII and *Sal* I sites of BS(−) (Stratagene). DNA fragments containing the insert regions were amplified by PCR using the M13mp19 universal and reverse sequencing primers (Pharmacia). *In vitro* run-off transcription of amplified DNA was as described (8).

Infection of *Chenopodium quinoa* Protoplasts. *Ch. quinoa* protoplasts were prepared and inoculated with circular plasmid DNA (25 μg) by electroporation as described (8) except that a high-voltage pulse of 750 V/cm was applied.

Agroinfection. The BWYV cDNA flanked by the control sequences of pBW.RN and pBW.N was moved into pBin19 (16) on a *Kpn* I–*Sal* I fragment (pBinBW.RN and pBinBW.N, respectively). Recombinant vectors were introduced into *A. tumefaciens* LBA4404 by electroporation (17) or by triparental mating (18). The chromosomal background of the resulting transformants is LBA4404 (pAL4404::pBinBW.N) and LBA4404 (pAL4404::pBinBW.RN). For agroinfection, the *A. tumefaciens* in a 48-hr culture were collected by centrifugation and resuspended in 1/100th volume of 20 mM Tris Cl at pH 8. *Physalis floridana* (six-leaf stage), *Nicotiana benthamiana* (six- to eight-leaf stage), and *Capsicum annuum* (four-leaf stage) were inoculated by making several incisions of 1 mm depth in the stem and applying 20 μl of inoculum (containing ≈10^9 bacteria) to the cuts. Inoculation of *Nicotiana clevelandii* (6- to 8-cm diameter) was performed by using a Hamilton syringe to inject a total dose of 20 μl of inoculum at about 10 sites in the leaf midribs and petioles.

Aphid Transmission Tests. Aphids (*Myzus persicae*) were reared on healthy *Ca. annuum*. Apterous adults and fourth-instar larvae were deposited on excised leaves of agroinfected plants in small plastic boxes. On *N. benthamiana*, aphid survival was greatly improved by first washing the leaves with 2% DDN150 (Franklab, Montigny-le-Bretonneux, France) and then with water. After a 1- to 2-day acquisition period, the aphids were transferred to healthy *Montia perfoliata* (approximately five aphids per plant). The aphids were killed by insecticide treatment after 5 days, and virus detection by ELISA was performed 3 weeks later.

Detection of Virus Coat Protein and RNA. Infection after agroinoculation and aphid-transmission experiments was tested by double-antibody sandwich ELISA (19) using BWYV-specific antisera. In each set of experiments, the threshold for infection was defined as the mean ELISA absorbance value for four healthy plants plus three times the standard deviation of the healthy plant values (19). Total RNA was extracted from protoplasts (8) 72 hr after electroporation or from randomly selected leaves of plants (20) 3–6 weeks postinoculation, and viral RNA was detected by Northern hybridization using a ^{32}P-labeled antisense RNA probe (8). Electron microscopic detection of virus in extracts of agroinfected plants was as described (21).

The 3′ termini of progeny viral RNA were analyzed by RNase mapping (22) using radioactive antisense RNA probes made by transcribing *Hin*dIII-linearized p3′BW.RN or p3′BW.N. Dried RNA samples (5–10 μg of total plant RNA, 2–4 μg of total protoplast RNA, 10–30 ng of transcript) were dissolved in 30 μl of hybridization buffer with 10^6 cpm of probe, allowed to hybridize, and then treated with RNase as described (22). RNase-resistant products were sized by electrophoresis through a 6% polyacrylamide/8 M urea sequencing gel.

RESULTS AND DISCUSSION

Plasmids for Agroinfection. In previous agroinfection experiments it has generally proven necessary to insert tandem copies of the viral genome into the T-DNA (for review, see ref. 11). For the geminiviruses and CaMV, which have DNA genomes, the viral sequence is probably liberated from the T-DNA by homologous recombination between the sequence repeats, but, in the case of CaMV, escape can also involve synthesis of the genome-plus length 35S transcript, an intermediate in DNA replication (23). The mechanism by which a viroid sequence might escape and begin autonomous replication is not known.

In this paper we have devised an agroinfection strategy for delivery of a virus with a linear RNA genome. Evidently, a

plus-strand RNA virus such as BWYV with no DNA intermediate in its replication cycle is not expected to possess transcription signals active at the DNA level. Consequently, we have provided exogenous transcription initiation and termination signals flanking the cloned viral cDNA. The CaMV 35S promoter, which is active in many plant tissues (24), was used to initiate transcription. The promoter cassette was placed so that the resulting transcript would have only one nonviral residue (guanosine) at its 5′ end (Fig. 1*A*).

To provide a transcription termination signal, a 333-nt DNA fragment containing the NOS 3′ noncoding sequence was inserted behind the cDNA to produce pBW.N (Fig. 1*A*). Viral RNA transcripts terminating *in planta* at the predicted site will possess a 3′ nonviral extension of 156 nt derived from the NOS cassette plus a 3′ poly(A) tail. BWYV run-off transcripts with a nonviral 3′ extension of 32 nt are infectious in protoplasts (8). However, experience with other viral RNAs suggested that a very long 3′ nonviral extension might greatly diminish biological activity (25, 26). Therefore, an autocatalytic sequence (27), or "ribozyme," was placed just downstream of the viral RNA 3′ terminus (see *Materials and Methods*) to produce pBW.RN (Fig. 1*A*). The ribozyme (Fig. 1*B*) was modeled after the self-cleaving RNA sequences characteristic of avocado sunblotch viroid, tobacco ringspot virus satellite RNA, and many virusoids (28). These sequences can fold into a "hammerhead" structure, which, in the presence of Mg^{2+}, directs spontaneous autocleavage at a specific site on the RNA (13). Based upon the hammerhead model, the ribozyme in pBW.RN should cleave the primary transcript 1 nt downstream of the viral 3′ end (Fig. 1*B*). A similar approach has been used to eliminate nonviral sequences from the 3′ end of *in vitro* run-off transcripts of cloned brome mosaic virus cDNAs (26).

The activity of the ribozyme was tested by using an *in vitro* run-off transcript of p3′BW.RN, which contains the 3′ terminal portion of the BWYV sequence plus the ribozyme and NOS termination cassette. PAGE revealed that an estimated 50% of the transcript underwent spontaneous cleavage during transcription to yield two fragments of the size expected for ribozyme-induced scission (Fig. 2, lane 2). An antisense transcript of p3′BW.RN or a sense transcript of p3′BW.N (no ribozyme sequence) did not undergo cleavage (Fig. 2, lanes 1 and 3). We conclude that the ribozyme in p3′BW.RN cuts the primary transcript at or near the predicted site.

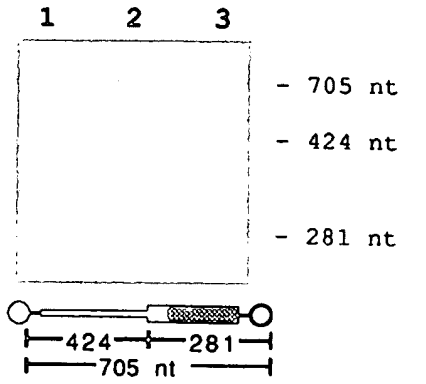

FIG. 2. Self-processing of a transcript containing the ribozyme sequence. ^{32}P-labeled sense RNA was transcribed from PCR-amplified fragments of p3′BW.RN (lane 2) and p3′BW.N (lane 3) with bacteriophage T3 RNA polymerase. ^{32}P-labeled antisense RNA of p3′BW.RN (lane 1) was transcribed with bacteriophage T7 RNA polymerase. After 30 min, the transcription reactions were stopped, and the products were analyzed by electrophoresis through a 6% polyacrylamide/8 M urea gel followed by autoradiography. Sizes of uncleaved transcript and expected cleavage products are indicated on the map (below) and beside the autoradiograph. ○, T3 promoter; ○, T7 promoter. Other symbols are as in Fig. 1.

Protoplast Infection Experiments. We have shown previously that transcripts of cloned BWYV cDNA are infectious to *Ch. quinoa* protoplasts (8). When circular pBW.RN and pBW.N plasmids were introduced into *Ch. quinoa* protoplasts by electroporation, both constructs were infectious as judged by the appearance of viral RNA (Fig. 3, lanes 2 and 3) in extracts of protoplasts harvested 72 hr postinoculation. The ability of pBW.N to initiate an infection suggests that the BWYV replicase can recognize and initiate minus-strand RNA synthesis on the viral 3′ sequence even when it is embedded in a longer transcript. Alternatively, RNA molecules capable of being replicated could be engendered by premature termination of transcription (or posttranscriptional cleavage) near the authentic viral RNA 3′ terminus of the primary transcript. A similar finding has been reported for tobacco transformed with tobacco mosaic virus cDNA under control of the 35S promoter but with no transcription terminator (12).

Agroinfection. Three known hosts for BWYV (30) were inoculated as described in *Materials and Methods* with *A. tumefaciens* carrying pBinBW.RN. With *P. floridana*, symptoms (leaf yellowing, leaf curling, and petiole necrosis) began to appear 10 days postinoculation. Early symptoms were less severe on *N. benthamiana* and *N. clevelandii*, with interveinal leaf yellowing becoming pronounced only 4–6 weeks after infection. Virus could be detected by ELISA 3 weeks postinoculation in segments of randomly selected leaves of 33–100% of the plants inoculated with pBinBW.RN in different experiments (Table 1). ELISA values for all the plants scored as infected in Table 1 were strongly positive, at least 5 times and often 10 times the infection threshold value as defined in *Materials and Methods*. Infection of *N. clevelandii* occurred with greater frequency than for the other two hosts (Table 1). It is not yet known if this difference reflects greater susceptibility of *N. clevelandii* to virus infection or if the inoculation procedure (jabbing with a syringe) was more efficient than that employed with the other two hosts. Agroinfection with *A. tumefaciens* carrying pBinBW.N rather than pBinBW.RN was also successful in *N. clevelandii* (Table 1).

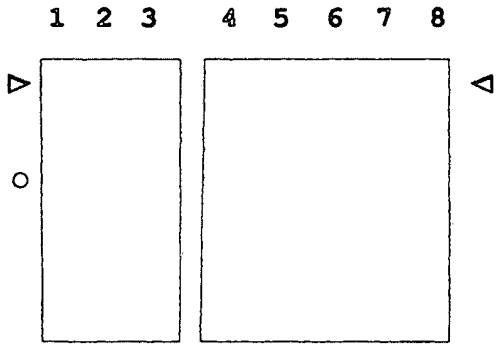

FIG. 3. Detection of BWYV RNA in total RNA from infected protoplasts and agroinfected *N. clevelandii* by Northern hybridization. RNA was extracted from protoplasts electroporated with the full-length transcript of pBW_0 (8) (lane 1), pBW.RN DNA (lane 2), and pBW.N DNA (lane 3). RNA was extracted from aphid-infected *P. floridana* (lane 4), a mock-inoculated *N. clevelandii* plant (lane 5), and three agroinfected *N. clevelandii* plants that tested positive for infection by ELISA (lanes 6–8). Triangles mark the position of BWYV genomic RNA, and the black dot marks the position of the major viral subgenomic RNA (8). The other minor bands in lanes 4–8 have not been characterized, but some may arise from ribosomal RNA shadowing (29). Although it has been detected in other experiments (data not shown), the subgenomic RNA is not readily visible against the background of minor bands in the whole plant extracts in lanes 6–8.

Table 1. Efficiency of transmission of BWYV by agroinfection

Host	Experiment	Inoculum*	Plants infected/ inoculated
P. floridana	1	RN	5/15
	2	RN	7/10
	3	RN	6/14
N. clevelandii	1	RN	8/10
	2	RN	11/12
	3	RN	5/6
	4	RN	4/4
		N	23/25
	5	RN	2/4
		N	18/26
N. benthamiana	1	RN	6/12
	2	RN	5/12
Ca. annuum	1	RN	0/11

*RN, inoculation with LBA4404 (pAL4404::pBinBW.RN); N, inoculation with LBA4404 (pAL4404::pBinBW.N).

Virus-like particles were readily detected by immunosorbent electron microscopy of extracts of agroinfected plants (data not shown). Viral RNA was detected by Northern hybridization of total RNA extracted from ELISA-positive agroinfected plants (Fig. 3, lanes 6–8) but was not detected in a mock-inoculated plant (Fig. 3, lane 5). Indeed, no viral infection was ever detected by ELISA for noninoculated control plants (n = 30) kept in the same growth chamber or for *P. floridana* (n = 24) inoculated with *A. tumefaciens* harboring pBin19 without a cDNA insert. Agroinoculation of *Ca. annuum*, a nonhost for BWYV isolate FL1 (H. Lot, personal communication) but a host for *A. tumefaciens* (31), gave no infection (Table 1). Thus, the failure of viruliferous aphids to transmit BWYV to *Ca. annuum* (a plant on which they feed readily) is apparently due to incompatibility between the virus and the plant and not to a deficiency in vector transmission, as it seems unlikely that a totally different inoculation route such as agroinoculation would suffer from the same deficiency.

To determine if naked BWYV cDNA (plus flanking control sequences) can initiate an infection, 14 *N. clevelandii* were each inoculated with 50 μg of purified pBW.RN plasmid using the syringe jabbing procedure employed for agroinoculation of *N. clevelandii*. This amount of pBW.RN corresponds to about 5000 times the amount of viral cDNA present in the *A. tumefaciens* inoculum used in the experiments shown in Table 1. None of the plants so inoculated became infected. We conclude that efficient delivery of the pBW.RN cassette to susceptible sites on whole plants requires the transfer functions provided by the *A. tumefaciens* Ti plasmid.

The fate of nonviral 3′ sequences after agroinfection was studied by RNase mapping (22). A ^{32}P-labeled RNA probe complementary to the NOS cassette, the ribozyme sequence, and the 3′ terminal portion of BWYV (nt 5367–5641) was produced by run-off transcription of *Hin*dIII-linearized p3′BW.RN. The antisense probe RNA was hybridized to progeny viral RNA from *N. clevelandii* agroinfected with pBinBW.RN and from transcript-infected protoplasts. The material was then treated with RNase under conditions designed to degrade single-stranded but not double-stranded RNA, and the RNase-resistant portion of the probe was sized by PAGE. Transcript of pBW_0 (8), which does not contain the ribozyme and NOS cassette, was carried through the same hybridization and RNase treatment to provide a mobility marker (predicted size, 274 nt) for the portion of the probe complementary to the viral 3′ terminal sequence. The mobility of this marker (Fig. 4, lane 8) was identical to that of the RNase-trimmed probe following hybridization with progeny RNA from infected protoplasts (lane 7) and from naturally infected (lane 6) and agroinfected (lane 5) plants, indicating that successful multiplication of the virus following agroinoculation is accompanied by elimination of the 3′ nonviral sequences. Similar experiments using progeny RNA from a pBinBW.N agroinfection showed that the downstream nonviral sequences predicted for the primary transcript are likewise absent from the progeny RNA (Fig. 4, lane 11). Thus, to achieve agroinfection with BWYV, no special precautions are needed to generate an authentic or nearly authentic viral RNA 3′ terminus *in planta*. This may not, however, be the case for other luteoviruses.

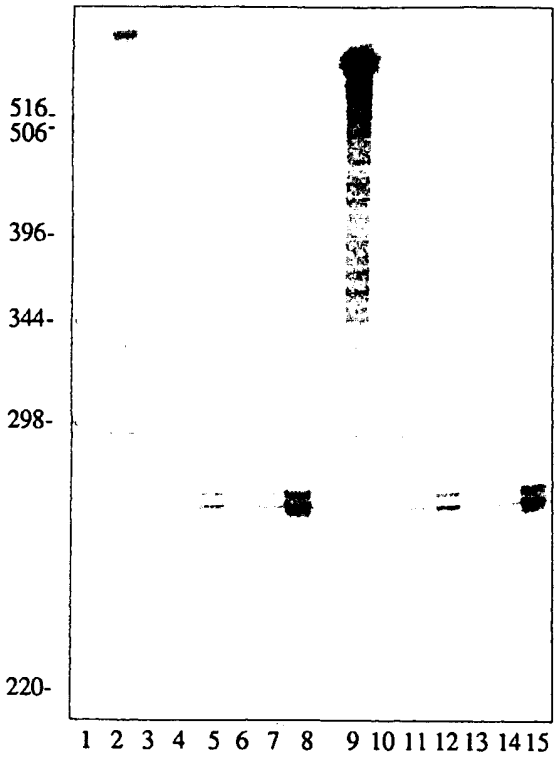

FIG. 4. RNase protection analysis of the 3′ termini of progeny viral RNA from plants agroinfected with pBW.RN and pBW.N. RNA samples were from healthy *N. clevelandii* (lanes 3 and 10), *N. clevelandii* agroinfected with pBW.N (lanes 4 and 11) or with pBW.RN (lanes 5 and 12), naturally BWYV-infected *P. floridana* (lanes 6 and 13), transcript-infected protoplasts (lanes 7 and 14), and pBW_0 transcript (lanes 8 and 15). The RNA was hybridized with ^{32}P-labeled antisense probe from p3′BW.RN (lanes 3–8) or p3′BW.N (lanes 10–15) prior to RNase treatment. Lane 1, single-stranded DNA size markers; lane 2, full-length p3′BW.RN probe; lane 9, full-length p3′BW.N probe. Mobility was determined by electrophoresis through a denaturing 6% polyacrylamide gel and autoradiography. The two heavily labeled bands in lanes 4–8 and 11–15 differ by 4 nt in mobility. The shorter of these two species and most of the other minor bands may arise from overdigestion of the viral RNA–probe hybrid.

To test the transmissibility of virus in agroinfected plants, nonviruliferous *My. persicae* were allowed to feed on such plants and were then transferred to healthy *Mo. perfoliata*. Successful aphid transmission of the virus, as assayed by ELISA, produced typical symptoms and occurred at a high rate for all three types of agroinfected source plants (10 of 10, 12 of 12, and 15 of 24 *Mo. perfoliata* infected for transmission tests from *P. floridana*, *N. benthamiana*, and *N. clevelandii*, respectively). We conclude that the viral sequence transmitted by agroinfection is still fully competent for transmission by aphids and for symptom development.

Concluding Remarks. Cloned cDNA of several mechanically transmitted RNA viruses, when positioned behind the

35S promoter, have been shown to be infectious by mechanical inoculation to plants (32–34). The agroinfection experiments described in this paper differ from these findings in that they offer a means of obtaining an infection starting with cDNA of a virus with a linear RNA genome, which cannot be transmitted by mechanical inoculation of virus, viral RNA, or viral cDNA (plus transcription control sequences). Presumably, such an approach can be applied to virtually any plus-strand RNA plant virus but will evidently be of particular value for viruses such as BWYV for which no other means of delivery is presently available. In particular, we can now envision use of site-directed mutagenesis to map BWYV genes governing viral cell-to-cell movement, virus–aphid interactions, host range, and symptom expression.

The authors would like to thank H. Lot (Institut National de la Recherche Agronomique, Montfavet) for advice and BWYV antiserum. The original 35S promoter construct (pCaP35J) was obtained from J. Yamaya (Kirin Brewery, Kitsuregawa-Machi Tochigi-Ken, Japan). R.-M.L. received fellowships from the Organisation for Economic Cooperation and Development (Paris) and the Ministry of Science and Research of Land Sachsen-Anhalt, Magdeburg, F.R.G. (LSA-21). Agroinfection experiments were carried out under the authorization of the Genetic Engineering Commission of the Ministère de la Recherche et de la Technologie.

1. Casper, R. (1988) in *The Plant Viruses: Polyhedral Virions with Monopartite RNA Genomes*, ed. Koenig, R. (Plenum, New York), Vol. 3, pp. 235–258.
2. Martin, R., Keese, P. K., Young, M. J., Waterhouse, P. M. & Gerlach, W. L. (1990) *Annu. Rev. Phytopathol.* **28**, 341–363.
3. Miller, W. A., Waterhouse, P. M. & Gerlach, W. L. (1988) *Nucleic Acids Res.* **16**, 6097–6111.
4. Veidt, I., Lot, H., Leiser, M., Scheidecker, D., Guilley, H., Richards, K. & Jonard, G. (1988) *Nucleic Acids Res.* **16**, 9917–9932.
5. Mayo, M. A., Robinson, D. J., Jolly, C. A. & Hyman, L. (1989) *J. Gen. Virol.* **70**, 1037–1051.
6. Van der Wilk, F., Huisman, M. J., Cornelissen, B. J. C., Huttinga, H. & Goldbach, R. (1989) *FEBS Lett.* **245**, 51–56.
7. Young, M. J., Kelly, L., Larkin, P. J., Waterhouse, P. M. & Gerlach, W. L. (1991) *Virology* **180**, 372–379.
8. Veidt, I., Bouzoubaa, S. E., Leiser, R. M., Ziegler-Graff, V., Guilley, H., Richards, K. & Jonard, G. (1992) *Virology* **186**, 192–200.
9. Zambryski, P., Herrera-Estrella, L., De Block, M., Van Montagu, M. & Schell, J. (1984) in *Genetic Engineering: Principles and Methods*, eds. Setlow, J. & Hollaender, A. (Plenum, New York), Vol. 6, pp. 253–278.
10. Grimsley, N., Hohn, B., Hohn, T. & Walden, R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3282–3286.
11. Grimsley, N. H. (1990) *Physiol. Plant.* **79**, 147–153.
12. Yamaya, J., Yoshioka, M., Meshi, T., Okada, Y. & Ohno, T. (1988) *Mol. Gen. Genet.* **211**, 520–525.
13. Haseloff, J. & Gerlach, W. L. (1988) *Nature (London)* **334**, 585–591.
14. Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J. & Steinbiss, H. H. (1987) *Nucleic Acids Res.* **15**, 5890.
15. Bevan, M., Barnes, W. & Chilton, M. D. (1983) *Nucleic Acids Res.* **11**, 369–385.
16. Bevan, M. (1984) *Nucleic Acids Res.* **12**, 8711–8721.
17. Mozo, T. & Hooykaas, P. J. J. (1991) *Plant Mol. Biol.* **16**, 917–918.
18. Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7347–7351.
19. Herrbach, E., Lemaire, O., Ziegler-Graff, V., Lot, H., Rabenstein, F. & Bouchery, Y. (1991) *Ann. Appl. Biol.* **118**, 127–138.
20. Bouzoubaa, S., Ziegler, V., Beck, D., Guilley, H., Richards, K. & Jonard, G. (1986) *J. Gen. Virol.* **67**, 1689–1700.
21. Derrick, K. S. (1973) *Virology* **56**, 652–653.
22. Zinn, N., Dimaio, D. & Maniatis, T. (1983) *Cell* **34**, 865–879.
23. Grimsley, N., Hohn, T. & Hohn, B. (1986) *EMBO J.* **5**, 641–646.
24. Benfey, P. N., Ren, L. & Chua, N. H. (1989) *EMBO J.* **8**, 2195–2202.
25. Dawson, W. O., Beck, D. L., Knoor, D. A. & Grantham, G. L. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1832–1836.
26. Dzianott, A. M. & Bujarski, J. J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4823–4827.
27. Forster, A. C. & Symons, R. H. (1987) *Cell* **49**, 211–220.
28. Symons, R. H. (1989) *Trends Biochem. Sci.* **14**, 445–450.
29. Palukaitis, P., Garcia-Arenal, F., Sulzinski, M. A. & Zaitlin, M. (1983) *Virology* **131**, 533–545.
30. Peters, D. (1988) in *Virus Yellows Monograph: A Synopsis of Research Carried out on Beet Yellowing Viruses and Their Vectors in Europe*, ed. Winner, C. (Inst. Int. Rech. Betteravières Pests and Diseases Study Group, Brussels), pp. 87–115.
31. De Cleene, M. & De Ley, J. (1976) *Bot. Rev.* **42**, 389–466.
32. Mori, M., Mise, K., Kobayashi, K., Okuno, T. & Furusawa, I. (1991) *J. Gen. Virol.* **72**, 243–246.
33. Commandeur, U., Jarausch, W., Li, Y., Koenig, R. & Burgermeister, W. (1991) *Virology* **185**, 493–495.
34. Maiss, E., Timpe, U., Brisske-Rode, A., Lesemann, D.-E. & Casper, R. (1992) *J. Gen. Virol.* **73**, 709–713.